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04AUG03 E827252-1 000056
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VACCINE

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VACCINE

The present invention relates to a novel peptide-based vaccines, uses of such vaccines in prophylactic and therapeutic treatment of human and animal diseases, such as viral infection and cancer.

Most of the successful vaccines depend on neutralising antibodies raised by classic attenuated or killed pathogens. However, for pathogens causing chronic infection-such as HIV, hepatitis C virus, mycobacteria and parasites – or in the case of cancer, a T-cell mediated immune response is crucial. Molecular understanding of MHC antigen presentation and the T-cell immune responses led to the use of defined antigenic peptide plus cytokines and/or co-stimulatory molecules in attempts to develop vaccines. One of the basic problems in all these attempts was the difficulty to reconstitute an antigen delivery system that is qualitatively and quantitatively similar to antigen presenting cells (APC) *in vivo*.

CD8+ cytotoxic T lymphocytes (CTL) recognise antigens as small antigenic peptides that assemble with major histocompatibility complex (MHC) class I molecules. The antigenic peptides are generated in the cytosol of APC and subsequently translocated into the lumen of the endoplasmic reticulum (ER) (Rock, K. L. & Goldberg, A. L. *Annu Rev Immunol* **17**, 739-779 (1999)). The MHC class I heavy chain is synthesised and inserted into the lumen of the ER and where it forms a dimer with b2-microglobulin (b2M) (Natarajan *et al Rev Immunogenet* **1**, 32-46 (1999); Pamer E, & Cresswell P, *Annu Rev Immunol.* **16** 323-358 (1998)). The dimers are retained in the ER until they assemble with proper antigenic peptides. The process of MHC class I dimer and assembly with peptides in the ER is catalysed by chaperones such as BIP, calnexin, calreticulin, and Erp57 (Paulsson K, & Wang P., *Biochim Biophys Acta.* **1641**(1) 1-12 (2003)).

The assembled MHC class I are rapidly expressed on the cell surface of APC, such as infected or malignant cells. The recognition of peptide-MHC class I by T cell receptor leads the CTL to kill target cells expressing infectious or tumor antigens.

Following the identification of CTL recognized epitopes from viral or cancer proteins, synthetic peptide-based vaccines designed to elicit T-cell immunity became an attractive approach to the prevention or treatment of infectious and malignant diseases (Furman MH, & Ploegh HL., *J Clin Invest.* **110** (7) 875-9 (2002); Berinstein N. *Semin Oncol.* **30** (3) (Suppl 8), 1-8 (2003); Falk *et al Nature* **348**, 248-251. (1990); (Van Bleek GM, & Nathenson SG., *Nature* **348**: 213-216 (1990); Kast, W.M., & Melief, C.J. *Immunol. Lett.* **30**:229-232 (1991)). There are a number of different forms of peptide vaccines based on these delivery systems. The simplest form is peptides dissolved in aqueous solutions. Direct injection of soluble antigenic peptides was shown to be unsuccessful at stimulating CTL responses, either because of their rapid biodegradation or induction of T cell anergy resulting from the antigenic stimulation by immature APC (Kyburz, D. *et al. Eur. J. Immunol.* **23**:1956-1962 (1993); Toes, R.E *et al Proc. Natl. Acad. Sci. USA.* **93**:7855-7860 (1996); Amoscato *et al J. Immunol.* **161**, 4023-4032 (1998)). An additional complication reported from the use of synthetic peptide-derived vaccines is the induction of CTLs that, while they are capable of killing target cells that are exogenously pulsed with peptide, they are not able to recognise target cells that naturally process and present the peptide epitope, such as infected or malignant cells (Dutoit, V. *et al. J. Clin. Invest.* **110**:1813-1822 (2002)).

It has been reported that MHC class I antigen presentation is qualitatively controlled in the ER for selecting correct peptides. Only the correctly assembled MHC class I could express on the surface of APC. The use of adjuvants did little to increase the presentation quality of synthetic peptides (Schijns, V.E. 2001. *Crit. Rev. Immunol.* **21**:75-85 (2001). An improved version of the peptide-vaccine has been constructed as an artificial lipo-membrane (BenMohamed *et al Lancet Infect Dis.* **2**(7), 425-31 (2002)) with peptide-loaded recombinant MHC class I. Although liposome strategy is able to incorporate peptide bound MHC class I molecules in the lipid membrane before injection into patients, the sophisticated loading system in the ER of APC could not be easily imitated by a simple mixture of recombinant MHC class I, synthetic peptide and liposomes. Only a few peptides would assemble with

recombinant MHC class I *in vitro* (Ostergaard Pedersen L, *et al Eur J Immunol.* **31**(10), 2986-96 (2001).

In addition, the incorrect orientation of inserted MHC class I and lack of co-stimulatory molecules made it difficult to induce effective immune responses. Since the professional APCs have the unique ability of presenting optimal antigen and for initiating a cellular immune response by naïve T cells, strategies are being developed to generate autologous dendritic cells (DC), a key APC, as vaccine vehicles *ex vivo* (Banchereau, J. *et al. Annu. Rev. Immunol.* **18**:767-811 (2000)). Initial studies showed that antigenic peptide-pulsed DC used as vaccines *in vivo* could induce a CTL response (Tsai, V. *et al. J. Immunol.* **158**:1796-1802 (1997)). Despite the positive evidence reported from a number of human clinical trials, there is no biochemical evidence showing that the pulsed peptides are indeed loaded on the surface MHC class I, which questions the efficiency of peptide-pulsed APCs to induce effective immune responses.

There is therefore a need for a vaccine preparation that can overcome these problems and present a therapeutically effective alternative to conventional vaccines. Such vaccines should resemble the quality of the endogenous presented antigen by APC cells while preserving high efficacy and avoiding side effects.

According to a first aspect of the invention, there is provided a composition comprising an isolated microsome of the endoplasmic reticulum of an animal cell, or a membrane fragment thereof, in association with an externally disposed peptide antigen and a protein of the Major Histocompatibility Complex (MHC).

The ER derived microsomes contain both MHC class I and class II molecules (Bryant *et al Adv Immunol.* **80**, 71-114 (2002)). The present invention is equally applicable with respect to the MHC class I restricted antigenic peptides as well as the class II molecules as well. The protein of the MHC in the composition may be from a heterologous source with respect to the cell from which the microsomes are obtained.

The MHC family of proteins are encoded by the clustered genes of the major histocompatibility complex (MHC). MHC molecules are expressed on the cells of all higher vertebrates. They were first demonstrated in mice and called *H-2 antigens* (histocompatibility-2 antigens). In humans they are called *HLA antigens* (human-leucocyte-associated antigens) because they were first demonstrated on leucocytes (white blood cells). Class I and class II MHC molecules are the most polymorphic proteins known - that is, they show the greatest genetic variability from one individual to another - and they play a crucial role in presenting foreign protein antigens to cytotoxic and helper T cells, respectively. Whereas class I molecules are expressed on almost all vertebrate cells, class II molecules are restricted to a few cell types that interact with helper T cells, such as B lymphocytes and macrophages. Both classes of MHC molecules have immunoglobulin-like domains and a single peptide-binding groove, which binds small peptide fragments derived from foreign proteins. Each MHC molecule can bind a large and characteristic set of peptides, which are produced intracellularly by protein degradation. After they form inside the target cell, the peptide-MHC complexes are transported to the cell surface, where they are recognized by T cell receptors. In addition to their antigen-specific receptors that recognize peptide-MHC complexes on the surface of target cells, T cells express CD4 or CD8 co-receptors, which recognize non-polymorphic regions of MHC molecules on the target cell: helper cells express CD4, which recognizes class II MHC molecules, while cytotoxic T cells express CD8, which recognizes class I MHC molecules. (Alberts *et al*, "*Molecular Biology of the Cell*", 3rd edition, 1229-1235 (1994)).

All eucaryotic cells have an endoplasmic reticulum (ER). Its membrane typically constitutes more than half of the total membrane of an average animal cell. It is organized into a netlike labyrinth of branching tubules and flattened sacs extending throughout the cytosol. The tubules and sacs are all thought to interconnect, so that the ER membrane forms a continuous sheet enclosing a single internal space. This highly convoluted space is called the ER lumen or the *ER cisternal space,* and it often occupies more than 10% of the total cell volume. The ER membrane separates the ER

lumen from the cytosol, and it mediates the selective transfer of molecules between these two compartments.

The ER plays a central part in lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles, including the ER itself, the Golgi apparatus, lysosomes, endosomes, secretory vesicles, and the plasma membrane. The ER membrane also makes a major contribution to mitochondrial and peroxisomal membranes by producing most of their lipids. In addition, almost all of the proteins that will be secreted to the cell exterior - as well as those destined for the lumen of the ER, Golgi apparatus, or lysosomes - are initially delivered to the ER lumen (Alberts *et al*, *"Molecular Biology of the Cell"*, 3rd edition, 577-595 (1994)).

Compositions in accordance with the present invention may be optionally formulated with an appropriate adjuvant, and/or cytokines that promote T-cell responses.

Microsomes in the context of the present invention are the cell free membrane vesicles of the endoplasmic reticulum (ER) compartment of any animal cell able to present antigenic peptide by means of the Major Histocompatibility Complex (MHC). The definition of ER-derived microsomes is based on the presence of so-called "ER-markers" which are proteins normally resident in the ER, such as BIP, p58, calnexin, calreticulin, tapasin.

The microsomes contained in a composition of the present invention can be isolated by any convenient means. Suitable methods include those of Saraste *et al* and/or Knipe *et al* (Saraste *et al Proc. Natl. Acad. Sci. U. S. A.* **83**, 6425-6429 (1986) and Knipe *et al J. Virol.* **21**, 1128-1139 (1977)). Such methods comprise homogenisation of cells or tissues, followed by separation of the cell nucleus by centrifugation at 7500rpm for 10 minutes, then recovering the "rough" microsomes by centrifugation at 15500 rpm for 54 minutes. "Rough" microsomes are microsomes that have ribosomes attached. The resuspended "rough" microsomes are then further purified by centrifugation through a sucrose cushion for differential centrifugation at 110,000g

for 60 minutes. The rough microsomes were subfractionated by further centrifugation at 37,000rpm for 10 hours on a sucrose gradient (to reach isopyknic conditions), and the ER containing fractions determined by Western blotting with appropriate antibody, for example anti-p58 antibody.

The animal cell from which the isolated microsome population is to be prepared can be any generally convenient cell type that has MHC molecules expressed by the cell. For example, cells of the blood or of the immune system such as, B-cells and macrophages, the so-called antigen presenting cells (APCs). However, cell types could also be used from tissues such as liver, kidney, lung, brain, heart, skin, bone marrow, pancreas etc.

The cells may be of a human or of a non-human animal. Suitably, the animal is a mammal. The animal may be a rodent species, e.g. a mouse, a rat or a guinea pig, or another species such as rabbit, or a canine or feline, or an ungulate species such as ovine, porcine, equine, caprine, bovine, or a non-mammalian animal species, e.g. an avian (such as poultry, e.g. chicken or turkey).

The cells from which the microsomes are prepared may be a cell line in culture. The cell line may be an immortalised cell line. The cell line may be ultimately derived from a non-embryonic tissue source.

In certain embodiments of the invention, the source of cells may be a genetically modified source of animal cells, such as a cell line, or a transgenic non-human animal. The cells or tissue from which the microsomes are prepared may be a humanised animal tissue or cell from a transgenic non-human animal whose genome has been modified by the insertion of one or more human genes.

In embodiments of the invention relating to microsomes prepared from a transgenic non-human animal or transgenic cell line, the transgenesis is the introduction of an additional gene or genes or protein-encoding nucleic acid sequence or sequences. The transgene may be a heterologous gene or an additional copy of a homologous gene,

optionally under the control of a constitutive promoter or an inducible promoter. The transgenesis may be transient or stable transfection of a cell or a cell line, or an episomal expression system in a cell or a cell line.

However, it is in the field of human medicine, in which the compositions of this aspect of the invention are expected to find greatest application as vaccines. It is therefore preferred that the source of cells from which the microsomes are prepared has an MHC allotype that is compatible to the MHC of the recipient of the composition when used as a vaccine. A particularly preferred embodiment is the provision of cells from the ultimate recipient of the composition when used as a vaccine.

Alternatively, a suitable source of human cells may be from a cell line, for example a non-embryo derived cell-line, suitably a B-cell line such as cell line 221. Such cell lines may also advantageously not express proteins of the Major Histocompatibility Complex (MHC) type class I.

Cell line 221 is an example of such a MHC negative cell line. The absence of a native MHC class I expression in such cells permits the modification of the cell line to express MHC class I of any desired genotype. This may be particularly important in achieving the full immunising effects of the vaccine composition, since different human populations express different MHC proteins. In such compositions of the invention, the MHC protein may therefore be of a heterologous source with respect to the cell from which the microsomes are obtained.

Some of the MHC class I, like HLA A2, are expressed in more than 20% of the population. In circumstances where, a MHC negative cell line is used, one or more than one compatible MHC gene is transfected into the cell line by means of conventional gene transfer methods and the transgene is constructed into a expression vector. The expression cassette of expression construct normally includes standard promoter, such as CMV promoter, or elongation factor I promoter or actin promoter, enhancer, inserted transgene and the poly-A signal to achieve optimal expression.

Before transfection, the expression cassette will be isolated from the plasmid backbone to avoid the expression of bacterial plasmid genes in transfected cells. The sequences of the MHC class I cDNAs and genomic DNAs are published and available (www.ncbi.nlm.nih.gov/Genbank). A MHC class I transfectants Bank can be constructed by using MHC class I negative or selected MHC class positive cell lines to transfect most of the MHC class I genes, respectively. The selection of the expression cassette will be dependent on the optimal expression of the transgene.

In addition to transfecting selected MHC genes, it may also be desirable to transfect or co-transfect the cells with genes encoding co-stimulatory molecules such as B7 and/or the genes encoding cytokines such as IL-2. In the case of cytokines, the transgene will be fused with trans-membrane domain of CD2 or CD4 for targeting the cytokines into the ER membrane. In addition, in order to enrich the level of MHC, co-stimulatory molecules and membrane-bound cytokines in the ER, KDEL or other ER retention signalling (Nilsson T, & Warren G., *Curr Opin Cell Biol.***6** (4), 517-21 (1994)) will be tagged at the C-terminus of the transgenes for the retention of transgene products in the ER. The expression cassettes for these transgenes are similar to MHC class I transgenes.

In compositions according to the present invention, the microsome may comprise a membrane fragment thereof. Suitably, such membrane fragments may be prepared by the method comprising the use of detergents or repeated freeze-thawing or sonication to break the microsome structure. Such fragments may also be similarly loaded with peptide antigen to form a composition of the present invention.

In an alternative embodiment of this aspect of the invention, the microsomes may be loaded with antigen and then subjected to further processing so as to provide "inside-out" microsomes thus exposing the inner surface of the ER membrane.

The compositions of the present invention are provided with a microsome, or a fragment thereof, in association with an externally disposed peptide antigen that has been loaded into the microsome. The association may be such that the peptide

antigen is inserted in the membrane of the microsome such at least one epitope of the peptide antigen is exposed with respect to the outer membrane of the microsome.

The antigenic peptides may be introduced or loaded into the microsome by means of incubating the microsome with the peptide antigen in the presence of a nucleoside triphosphate (NTP), for example adenosine triphosphate (ATP) and NTP re-generation system. It appears that an NTP, such as ATP, facilitates the incorporation of the peptide antigen into the microsome through protein transporters located in the membrane of the microsome. Without wishing to be bound unnecessarily by theory, it appears that once the microsome is incubated with the peptide antigen in the presence of an NTP that the antigen is able to associate with MHC class I proteins already present in the membrane of the microsome. Alternatively, the antigenic peptides may also loaded into the microsomes after inside-out processing and in this case, the NTP is not required.

The antigenic peptide present in association with the microsome suitably has one or more epitopes. An epitope is the smallest part of an antigen recognisable by the combining site of an immunoglobulin. Therefore, any type of MHC binding peptides, natural or synthesized or artificially modified, is included.

The antigenic peptides may be from a source that is foreign, i.e. non-self, or self, i.e. an autoantigen. Foreign antigenic peptides may originate from virus, bacteria, yeast, fungi, protozoa, or other micro-organism (i.e. an infectious agent), or of higher life forms such as plants or animals. In some embodiments of the invention, the antigen may be an auto-antigen, for example an antigen expressed by a neoplastic cell or cell of a cancer tumour, a normal self-protein (in the case of an tolerising vaccine for an auto-immune disorder).

In some embodiments of the invention, it may be preferred to prepare the composition with an antigenic peptide of more than one type, or antigenic peptides having a sequence modified to increase immunogenicity . The cell may also be transfected prior to the preparation of the microsomes with more than one type of MHC

molecules which may be useful in the case of recipients of the compositions when used as vaccines who have more than one type of MHC allotype.

In a preferred embodiment of this aspect of the invention, there is provided a composition as defined above in which the ratio of antigen to MHC molecule in the microsome is optimal for the induction of a specific immunoresponse, for example in the range of from 0.1 to 1.5, preferably of from 0.2 to 1.2 or 0.5 to 1.0, and most preferably from 0.2-0.5 to 1.0. The amount of loaded antigenic peptides may be different according to the level of immune response induced.

According to the present invention, purified microsomes representing the endoplasmic reticulum in antigen-presenting cells (APC) can be used to load antigenic peptides on its MHC class I or II molecules. Results from *in vitro* and *in vivo* immunisation described herein show that peptide-loaded microsome elucidates much stronger responses than peptide-loaded APC measured by T cell proliferation and production of IL-2. By quantitating the amount of peptide-receptive MHC class I molecules, the receptive class I molecules on APC surface are below the radio-chemical detective limit. However, a significant amount of peptide bound MHC class I is detected in the microsome. In addition, a similar amount of co-stimulatory molecules, B7.1 and B7.2 is detected in microsomes in comparison to cell surface. Thus, the microsomes loaded with antigenic peptides represent an effective vaccine composition.

The present invention has found that more than 50% of the MHC class I molecules in the ER of APC are peptide receptive. By the process of "inside-out", the microsomes loaded with Kb specific OVA-peptide can induce T cell responses *in vitro* and *in vivo*. In contrast, the APCs pulsed with same peptide have much less ability to stimulate T cell responses. Given that the microsomes contain co-stimulatory molecules, the microsomes isolated from APCs represent promising vehicles for peptide vaccines in the future for a wide variety of diseases.

According to a second aspect of the invention, there is provided a vaccine composition comprising a composition according to the first aspect of the invention.

According to a third aspect of the invention, there is provided a composition according to the first or second aspects of the invention for use in medicine. This aspect of the invention therefore extends to a method of treatment or prophylaxis of a subject, comprising the step of administering to the subject a vaccine as defined above.

According to a fourth aspect of the invention, there is provided the use a composition as defined above in the preparation of a vaccine for the prophylaxis or treatment of a disease condition. The disease may be an infection caused by a micro-organism or virus, or it may be a cancer which is characterised by neoplastic cell growth and/or tumour formation. Alternatively, the disease may be an autoimmune condition, such as rheumatoid arthritis, where a vaccine may have therapeutic use in inducing tolerance to self-antigens. Uses in accordance with this aspect of the invention also extend to methods of treatment of such disease conditions comprising administering said compositions to a subject in need thereof.

Defined antigenic peptides of major diseases can be readily selected from the scientific literature or identified by bioinformatic tools, (Renkvist *et al Cancer Immunol Immunother* **50**, 3-15 (2001); Coulie *et al* Immunol Rev **188**, 33-42 (2002); De Groot *et al Vaccine* **19** (31), 4385-95 (2001)). Possible diseases include viral infection, such as HIV infection, herpes virus infection, hepatitis C virus infection, cancer, such as melanoma, leukaemia, breast cancers and parasite infections, such as protozoan parasite infection of *Plasmodium*, the causative agent responsible for malaria, for example *Plasmodium falciparum, Plasmodium vivax, Plasmodium berghei, Plasmodium yoelii* or *Plasmodium knowlesi*, or another parasite such as *Toxoplasma gondii*, or *Trypanosoma brucei*, or *Entamoeba histolytica*, or *Giardia lambia*, or bacterial infection, such as *E. coli 0157, Vibrio cholerae,* etc. Auto-immune diseases include multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis.

In uses or methods in accordance with the third or fourth aspects of the invention, the compositions may be co-administered with one or more cytokines that can promote T cell immune response such as Il-2, IL-15, IL-6, GM-CSF, IFNγ, other cytokines promoting T cell responses, and/or conventional adjuvant. These can be suitably mixed with the microsomes loaded with antigen prior to administration.

In addition, the such methods may include the steps of detecting and monitoring the specific immune responses towards the vaccine, for example by techniques such as ELISA for detection of serum IL-2 and/or IFNγ, or an *in vitro* T cell response assay with peptide loaded microsomes, or a proliferative cell assay.

According to a fifth aspect of the invention, there is provided a process for the preparation of a vaccine composition as defined above, the process comprising incubating a microsome, a fragment thereof, or a processed "inside-out" microsome and an antigenic peptide in the presence of a nucleoside triphosphate, and formulating the resulting preparation in an physiological diluent and optionally an adjuvant. Reagents such as glucose have ability to preserve the conformation of prepared microsome vaccine may be included. Suitably, the incubated microsomes will be washed and resuspended in a vaccine solution of an physiological diluent containing contain amount of antigenic peptides for preventing the dissociation of MHC – peptide complex.

According to a sixth aspect of the invention, there is provided a kit of parts comprising a composition as defined above and one or more cytokine and/or adjuvant in sealed containers. Suitably, the kit will comprise instructions for use in a method or use of the invention as defined above.

According to a seventh aspect of the invention, there is provided a kit of parts comprising a composition as defined above and one or more cytokine and/or adjuvant molecules for separate, subsequent or simultaneous administration to a subject.

Preferred features for the second and subsequent aspects of the invention are as for the

first aspect *mutatis mutandis*.

In a particularly preferred embodiment of the invention there is provided a vaccine composition for the prophylaxis or treatment of a disease that can be characterised by the expression of a defined antigen or a peptide sequence which is potentially immunogenic by an infectious agent or which is characterised by the expression of an antigen of a native cell, in which the composition is prepared by:

(1) obtaining a sample of antigen presenting cells which express MHC proteins;

(2) homogenising the cells under conditions such that a preparation of microsomes is isolated;

(3) preparation of antigen, for example by means of recombinant DNA technology, or from isolation from a natural tissue source, or source of infectious agent, or in most cases synthesised antigenic peptides.

(4) incubation of antigenic peptides and microsome in the presence of an NTP or of antigenic peptides and inside-out processed microsomes to load microsome with antigenic peptides;

(5) formulation of loaded microsome as a vaccine in a physiological diluent and/or adjuvant as appropriate

As described above, the microsomes may also be prepared from an isolated population of cells or a cell line. The cells may be MHC negative so as to permit transfection of the cell line with appropriate nucleic acid encoding the MHC class molecule of choice for the vaccine. Preparation of antigen may also include synthesis of antigenic peptides by means of chemical means.

The invention will now be further described by way of reference to the following Examples and Figures which are provided for the purposes of illustration only and are not to be construed as being limiting on the invention. Reference is made to a number of Figures in which:

FIGURE 1 shows crosslinking of H2-Kb molecules by crosslinker-modified OVA peptide in the microsomes of RAW309Cr.1 cells. The ^{125}I-labeled ANB-NOS-OVA peptide was mixed with the microsomes of RAW309Cr.1 cells in the presence or absence of ATP-regenerating system or of native OVA-peptide at a ten-fold molar excess. The crosslinked H-2Kb was indicated.

FIGURE 2 shows concentration of OVA-peptide receptive H-2Kb in the microsomes of RAW309Cr.1 cells. For semi-quantitation of OVA-peptide receptive H-2Kb, 10 nMs of labelled peptide was incubated with the microsomes or RAW309Cr.1 cells, respectively, under the UV irradiation. The H-2 molecules were precipitated by R218 antiserum and crosslinked Kb molecules were quantitated by phospho-imaging.

FIGURE 3 shows detection of H-2 molecules in the microsomes or on the surface of RAW309Cr.1 cells. 30 μg proteins from NP40 lysates of RAW309Cr.1 microsomes or RAW309Cr.1 cells were separated on 10% SDS-PAGE. The lysates were diluted at the titration indicated and separated on the SDS-PAGE. Immunoblotting of H-2 molecules was detected by R218 anti-H-2 antiserum.

FIGURE 4 shows detection of B7.1, B7.2 and ICAM-1 in the microsomes of RAW309Cr.1 cells. 30 μg proteins from NP40 lysates of RAW309Cr.1 microsomes or RAW309Cr.1 cells were separated on 10% SDS-PAGE. Immunoblotting of B7.1, B7.2 and ICAM-1 was detected by specific antibodies.

FIGURE 5 shows stimulation of B3Z T cells by OVA-peptide edited microsomes. Microsomes from 2 x 10^5 RAW309Cr.1 cells were used to load OVA or Ld-specific peptide as described in Material and Methods. 2 x 10^5 RAW309Cr.1 cells were pulsed with OVA peptide (see Material and Methods). After washing, peptide-pulsed 2 x 10^5 RAW309Cr.1 cells, OVA-

loaded microsomes, Ld-peptide loaded microsomes, and the microsomes without peptide were co-cultured with 10^5 B3Z cells for over night. A) After washing with PBS, LacZ activity in B3Z cells was assayed by total cellular lysates with the LacZ substrate ONPG. The absorbance (415 nM) was read after incubation for four hours at 37°C. B3Z cells cultured with 100 nM OVA peptide and normal medium were used as positive and negative control for the B3Z stimulation. B) The supernatants of these cultures were submitted for measuring IL-2 production by ELISA. The experiment was repeated four times with similar results. Error bars indicate the SEM of triplicate cultures.

FIGURE 6 shows stimulation of B3Z T cells by the microsomes of 2 x 10^5 RAW309Cr.1 cells pre-loaded with different concentrations of OVA-peptides. Microsomes loaded with OVA-peptide at different concentration indicated were co-cultured with B3Z cells overnight before the assay of LacZ activity.

FIGURE 7 shows OVA-peptide edited microsomes stimulates specific T cell responses *in vivo*. C57BL/6 (H-2b) mice were primed i.s. by OVA-edited microsomes or Ld-peptide loaded microsomes or OVA peptide or OVA-pulsed RAW309Cr.1 cells and challenged by same stimulus after seven days. Six days after the challenge, enriched T cells were isolated from spleens and cultured at 10^5 cells/well with stimulus indicated. The RAW309Cr.1 cells were irradiated before co-culture with T cells. After three days, supernatants were harvested for cytokine ELISA (b) and cultures pulsed with [^{3}H]thymidine (a). The results are representative of groups of at least three mice per treatment group and the experiment was repeated four times with similar results. Error bars indicate the SEM of triplicate cultures. Similar set of experiments performed in Balb/c (H-2d) mice served as negative control.

FIGURE 8 shows activation of TCR induced MAK kinases. 10^7 T cells from OVA-microsomes immunised C57BL/6 were stimulated with OVA-pulsed RAW309Cr.1, OVA-microsomes and anti-CD3/CD28, respectively. Activation of ERK and JNK was detected by anti-p-ERK and anti-p-JNK

antibodies. Similar levels of ERK and JNK detected by anti-ERK and anti-JNK severed as loading control.

FIGURE 9 shows OVA-receptive H-2Kb detected in microsomes, but not on the surface of RAW309Cr.1 cells. The ^{125}I-labeled ANB-NOS-OVA peptide 10 nM was mixed without or with native OVA peptide at concentrations indicated. The mixed peptides were incubated with microsomes equivalent to 10^7 RAW309Cr.1 cells or with 10^7 RAW309Cr.1 cells. The crosslinked H2-Kb molecules were indicated.

Material and Methods

Cell lines and animals

B3Z is a CD8 T cell hybridoma that expresses LacZ in response to activation of T cell receptors specific for the SIINFEKL peptide presented by H-2Kb MHC class I molecules. RAW309Cr.1, a Kd/Kb murine macrophage cell line, used as APCs, was obtained from ATCC (ATCC TIB-69). All cells were cultured in Dulbecco´s modified Eagle's medium with 10% fetal calf serum. Female C57BL/6 mice H-2b and Balb/c mice H-2d were obtained at 6 weeks of age. All procedures with animals were carried out in accordance with approved protocols.

Antibodies, peptides, and peptide modification

All peptides were synthesised in a peptide synthesiser (model 431A, Applied Biosystems, Foster City, CA), using conventional F-moc chemistry, and were subsequently purified by HPLC. The purified peptides were dissolved in PBS.

Peptide OVA 257-264 (SIINFEKL) was modified by substitution of third residue isoleucine to tyrosine in order for iodination and by covalently coupling a phenylazide with a nitro group on the ε-amino group of lysine at position seven. This nitro group can be photoactivated. The crosslinker modification was performed by mixing 0.5 mg of ANB-NOS (N-5-azido-2-nitrobenzoyloxysuccinimide) in 200 µl DMSO with 100 µg peptide in 100 µl PBS and 50 µl CPAS (3-[cyclohexylamino]-1-propanesulfonic acid) (0.5 M, pH 10). The reaction was allowed to proceed for 30 min on ice. To

remove the excess ANB-NOS and ions, the mixture was purified by gel filtration on Sephadex G-10 and subsequently by HPLC. An aliquot (1μg) of the peptide was labelled by chloramines-T-catalyzed iodination (^{125}I). The modification and labelling experiments were performed in the dark.

Antisera, immunoprecipitation, and SDS-PAGE

Rabbit antiserum to H2 (R218) was kindly provided by Dr. Sune Kvist, Karolinska Institute. Monoclonal antibody specific to confirmed H2 (Y3) was kindly provided by Tim Elliot, Cambridge University. Antisera to JNK, ERK, p-ERK and p-JNK were obtained from (Santa Cruz Biotechnology). Immunoprecipitation, immunoblotting and SDS-PAGE were performed as described in Li *et al* (*J Biol Chem.* **274** (13), 8649-54 (1999)). Protein-A-Sepharose was obtained from Pharmacia (Uppsala, Sweden).

Example 1: Preparation of microsomes and peptide binding assay

Microsomes fromRAW309Cr.1, a Kd/Kb murine macrophage cell line were prepared and purified according to the procedure of Saraste *et al* (*Proc. Natl. Acad. Sci. U. S. A.* **83**, 6425-6429 (1986)).The immunogenetics of class I is Kb in RAW cells and Balb/c mice.

Preparation of microsomes from B cells based on a modification of Saraste *et al* (*Proc. Natl. Acad. Sci. U. S. A.* **83**, 6425-6429 (1986)) and Knipe *et al* (*J. Virol.* **21**, 1128-1139 (1977)) for fractionation of microsomal membranes was used. All steps were performed at 0-4 °C).

- 3x 10^9 cells are collected and washed once with cold PBS.
- Resuspend the cells in 20 ml STKMM-buffer with 10 μl of PMSF (100mM).
- Spin at 1500rpm for 5 min at 4°C.
- Resuspend in 10 ml H_2O (with 5μl PMSF).
- Homogenise in 40 ml Dounce, 20 strokes.
- Add 30 ml STKMM and mixing well.

- Pour over in JA-18 tubes.
- Centrifuge at 7500rpm for 10 min at 4°C.
- Carefully collect supernatant to the new tubes.
- Centrifuge at 15500rpm for 54 min at 4°C.
- Carefully wash the pellet with 10 ml STKMM buffer, then resuspend the pellet in 1 ml RM buffer with a pipette and homogenise in 15ml douncer. The rough microsomes will be diluted at a concentration of $A_{OD280} = 60$.
- Total microsomes (described above) were layered on top of 5 ml of 0.33 M sucrose containing 5 mM benzamidine, layered in turn on top of a sucrose cushion consisting of 1 ml of 2 M sucrose/5 mM benzamidine.
- Centrifugation in an SW41 rotor for 60 min at 110,000 × *g* yielded a total microsome band on top of the cushion. The total microsome band was carefully collected. Then, 2 M sucrose/5 mM benzamidine was slowly added to the microsomes to give a final concentration of 45% (w/v) sucrose.
- Microsomes were subfractionated by flotation using a modification of the method described in Paulsson *et al* (*J Biol Chem.* **277** (21), 18266-71 (2002)). 100 μl of the total microsomes in 3 ml of 45% (w/v) sucrose was placed at the bottom of an SW41 ultracentrifuge tube and overlaid with the following sucrose solutions: 1 ml of 30% and 1.9 ml each of 27.5%, 25%, 22.5%, and 20.0% (all solutions contained 5 mM benzamidine).
- After centrifugation at 4 °C for 10 h at 37,000 rpm (to reach isopyknic conditions), 25 fractions of 300 μl each were collected by upward displacement.
- The ER fractions will be determined by western blotting with anti-p58 antibody. (p58 is a ER protein).
- The poured ER fractions will be used in peptide-loading and immunisation experiments.

The cross-link mixture contained 50 or 100 nM (^{125}I)ANB-NOS-peptide and 10 µl of microsomes (60 A_{280}/ml) in a total volume of 100 µl RM buffer (250 mM sucrose, 50 mM TEA-HCl, 50 mM KOAc, 2 mM $MgOAc_2$, and 1mM DTT). After mixing, the

samples were immediately irradiated at 366 nm for 5 min at room temperature. The membranes were then recovered by centrifugation through a 0.5-M sucrose cushion in RM buffer. The membranes were washed once with cold RM buffer. The washing membranes were lysed for immunoprecipitation or for immune blotting. The crosslinking reaction with ATP contained an ATP regeneration system, described in Li *et al* (*J Biol Chem.* **274** (13), 8649-54 (1999)). The crosslinking of surface Kb molecules on RAW309Cr.1 cells was performed as mixing 100 nM (^{125}I)ANB-NOS-peptide with 10^7 cells, equivalent to amount of cells used for making 10 µl of microsomes in a total volume of 100 µl RM buffer. After mixing, the samples were immediately irradiated for 5 min at room temperature. The excess peptides were removed by washing with RM buffer. The cells were lysed for immunoprecipitation with Y3 antibody.

The detection of surface MHC class I was performed by incubating RAW309Cr.1 cells with Y3 antibody at 4°C for 15 min. After washing, the cells were lysed in 1% NP40 lysis buffer and the cleared lysates were precipitated with protein-A beads. The precipitated MHC class I were detected by immunoblotting with R218 antiserum.

The peptide-editing for stimulation of T cells was performed by mixing microsomes with native peptides with ATP regeneration system for 10 min at room temperature. The excess of peptides was removed after centrifugation through sucrose cushion in RM buffer. The loaded microsomes were repeatedly freeze/thaw alternately in liquid nitrogen and then in a water bath at 37°C, for 10 times. The processed microsomes were resuspended in PBS at concentration of (6 A_{280}/ml) and kept at -80oC until use. The peptide-pulsed RAW309Cr.1 cells was prepared as mixing peptides 100 nM with 10^7 cells in 1 ml medium over night or in 1 ml PBS for four hours at 37°C. The pulsed cells were either washed with PBS before mixing with B3Z T cells or add the mixture directly to the B3Z.

Example 2: Activation of B3Z T cell hybridoma

The prepared stimuli including peptide-edited microsomes, peptide-pulsed RAW309Cr.1 cells, OVA peptide, were added to culture of 10^5 B3Z cells in a total of

200 µl. Addition of PBS and anti-CD3/CD28 coated beads served as negative or positive control, respectively. After over night incubation, the activation of B3Z was represented by LacZ activity using o-nitrophenyl b-D-galactopyranoside (Sigma) substrate. The linear range of OVA-response was determined by the addition of serial dilutions of SIINFEKL to the medium due to that the B3Z cells themselves express Kb and present SIINFEKL.

Example 3: Detection of peptide-receptive MHC class I molecules in the microsome, but not on the surface of APCs.

An *in vitro* peptide transport and loading assay by using crosslinker modified peptides and isolated microsomes of the ER from RAW309Cr.1 has been reported (Li *et al J Biol Chem.* **274** (13), 8649-54 (1999)). The assay allows the examination of both the peptide translocation across the membrane of the ER in the presence of ATP and subsequently the peptide loading on MHC class I molecules (Wang *et al J Immunol.* **157** (1), 213-20 (1996)).

To detect the peptide-receptive MHC class I molecules in the microsomal membranes, a crosslinker (ANB-NOS) was conjugated to the ε-amino group of the lysine residue of an H2-Kb-binding ovalbumin (OVA) peptide (residues 257-264, SIIFEKL) and substituted the isoleucine at position 3 with tyrosine to allow for iodination. These modifications allowed photo-cross-linking of the OVA peptide to H2-Kb molecules during the assembly. For a quantitative comparison of peptide-receptive H2-Kb in microsomes and on cell surface of living RAW309Cr.1, the modified OVA peptide was labelled by ^{125}I and incubated with microsomes of RAW309Cr.1 and living RAW309Cr.1 cells under UV irradiation. Peptide-bound H2-Kb molecules were subsequently analysed by immunoprecipitation with an anti-H2 antibody Y3. In the absence of ATP, only a few Kb molecules were assembled with OVA peptides, while a significant amount of Kb molecules were cross-linked with OVA peptide in the presence of ATP (Fig. 1). This result confirms that a substantial amount of peptide receptive class I molecules exist in the ER.

A semi-quantitative analysis of OVA-crosslinked Kb in microsomes and on the surface of RAW309Cr.1 showed that in contrast to the high levels of peptide receptive Kb molecules in the microsomes, the OVA-bound Kb molecules on the surface of APCs was under the radio-chemical detective level (Fig. 2), suggesting again that peptide-receptive MHC class I molecules are mainly in the ER, but not on the surface of APCs. In a competing experiment, it has been shown that the binding of this modified OVA peptide to Kb is specific. In order to examine the affinity of the modified OVA-peptide, the labelled OVA peptide was competed by its native form at different concentrations. The native OVA peptide competed 50% of the report peptide at the concentration of report peptides and completely abolished binding at concentration of ten times of the report peptides (Fig. 2). Moreover, a Ld specific peptide could not compete the OVA binding. This shows that the binding affinity of modified OVA-peptide is Kb specific and similar to its native form. To quantitate the amount of peptides bound to Kb molecules in microsomes derived from 10^6 RAW309Cr.1, the labelled peptides were incubated with microsomes in the presence of ATP. After crosslinking, MHC class I were precipitated and dpm of peptide-bound Kb was measured and converted to the concentration of peptides. Results showed that about 500 to 1000 peptides were bound to Kb molecules in the microsomes of one cells. In addition, the amount of total MHC class I molecules in the ER are more than that on the surface of RAW309Cr.1 cells (Fig. 3). Thus, microsomes from APCs could be able to deliver sufficient peptide-MHC class I complexes to T cells.

Example 4: B7 and ICAM1 are presented in the microsomes of APCs.

A full T cell response requires signals from both antigen-MHC complex and co-stimulatory molecules such as B7 (Acuto *et al, Immunol Rev.* **192**, 21-31 (2003)). Like all the membrane proteins, co-stimulatory molecules are synthesised in the ER and subsequently expressed on the surface of APCs. To quantitate the amount of co-stimulatory molecules of B7 and ICAM-1 in the isolated microsomes, the microsomes equivalent to 5 x 10^6 RAW309Cr.1 were lysed and the clear lysates were analysed by western blotting with anti-sera specific to these molecules, respectively. In comparison, a total cell lysates of 5 x 10^6 RAW309Cr.1 were also blotted with same antibody. The intensity of B7.1, B7.2 and ICAM-1 bands was quantitated by density

analysis. Both B7 and ICAM-1 were readily detected in the microsomal samples (Fig. 4). The amount detected in microsomes was about half of the total cellular lysates. The presence of sufficient amount of co-stimulatory molecules in peptide-edited microsomes could mimic the functional surface of APCs for providing both antigen-MHC and co-stimulatory signals to T cells.

Example 5: Microsomes loaded with Kb-specific OVA peptides stimulate T cells *in vitro*

To investigate the ability for peptide-loaded microsomes to induce specific T cell response, the native OVA peptide-edited microsomes were processed for inside-out by repeated freeze-thaw method (materials and methods). The processed microsomes and OVA-peptide pulsed RAW309Cr.1 were used to stimulate B3Z T cell hybridoma which recognises Kb-SIINFEKL complex (Fremont *et al Proc Natl Acad Sci U S A.* **92** (7), 2479-83 (1995); Shastri N, & Gonzalez F., *J Immunol.* **150** (7), 2724-36 (1993)). After washing off the excessive peptide, OVA edited Microsome stimulated B3Z T cells by inducing IL-2 production and the expression of IL-2-promoter driven LacZ (Fig. 5). The specificity of OVA-Kb induced B3Z responses was supported by the unresponsiveness of B3Z cells to the microsomes without the peptide or loaded with Ld specific peptide (Fig. 5). Moreover, the levels of responses of B3Z to OVA-edited microsomes was correlated with the amount of OVA-peptides (Fig. 6). OVA-pulsed RAW309Cr.1 could induce the B3Z response in the presence of excessive peptides (Schott *et al Proc Natl Acad Sci U S A.* **99** (21), 13735-40 (2002)).

However, if excess peptides were removed by washing, the OVA-pulsed-RAW309Cr.1 could no longer induce B3Z response. Given that OVA itself could induce IL-2 production by B3Z cells (Fig. 5), suggests that not RAW309Cr.1, but OVA itself is the stimuli for B3Z. The ability of SIINFEKL to induce Kb restricted T cell responses *in vitro* has been reported recently, suggesting that CTL could present peptides to each other (Schott *et al Proc Natl Acad Sci U S A.* **99** (21), 13735-40 (2002)).However, the induction CTL response *in vitro* by peptide-edited microsomes, but not by peptide-pulsed RAW309Cr.1 is consistent with the peptide-binding results

(Fig. 2) and indicate that peptide-edited microsomes could mimic APCs to efficiently present antigenic peptides to TCR and stimulate full responses of CTLs.

Example 6: Microsomes loaded with Kb-specific OVA peptides induces OVA-peptide responses *in vivo*

To further examine the ability of OVA-edited microsomes to induce immune responses *in vivo*, OVA-edited microsomes from RAW309Cr.1 cells, microsomes loaded with Ld-specific peptide, soluble OVA peptides, RAW309Cr.1 pulsed with OVA peptides and PBS were used to induce immune response *in vivo*. Five groups of C57BL/6 or Balb/c mice, each group consisting of five mice, were injected twice subcutaneously with above stimuli, respectively. The interval between injections was one week. Six days after second injection, T cells were isolated from spleens and cross-stimulated *in vitro* with the five original stimuli, respectively. In addition, anti-CD3/CD28 coated beads were used as positive control. PBS stimulated T cells did not respond to any stimulation, while anti-CD3/CD28 induced proliferative responses in all the groups. OVA-peptide pulsed RAW309Cr.1 and microsomes loaded with Ld-specific peptide did not induce T cells responses (Fig. 7). In contrast, T cells from C57BL/6 groups of OVA-edited microsomes and OVA peptide responded to OVA-edited microsomes *in vitro*, but not to the OVA-pulsed RAW309Cr.1 or the microsomes loaded with Ld-peptides (Fig. 7). Compelling results from IL-2 production (Fig.7) again support that OVA-edited microsomes could induce specific T cells responses *in vivo* (Fig. 7). Balb/c has H-2d, therefore, there was not OVA response induced.

Example 7: TCR signalling pathways are activated by OV-microsomes

In order to analyse the TCR signalling in response to OVA-microsome stimulation, T cells isolated from C57BL/6 mice immunised by OVA-microsomes were used to induce TCR signalling *in vitro*. The activation of ERK and JNK was detected in the T cells stimulated with either anti-CD3/CD28 or with OVA-edited microsomes, but not with OVA-pulsed RAW309Cr.1 (Fig. 8). Thus, the biochemical evidence indicates a specific TCR signalling in response to OVA-Kb on microsomes and further supports

that microsomes edited with antigenic peptides could induce specific immune responses *in vivo*.

Discussion

These results demonstrate that the microsomes derived from the ER can be used to process edited antigenic peptides on MHC class I molecules and that the processed microsomes can reconstitute the functional surface of APCs to induce CTL responses. Thus, the antigenic peptide delivered by microsomal MHC class I in association with co-stimulatory molecules is a novel form of peptide vaccines.

CLAIMS

1. A composition comprising an isolated microsome of the endoplasmic reticulum of an animal cell, or a membrane fragment thereof, in association with an externally disposed peptide antigen and a protein of the Major Histocompatibility Complex (MHC).

2. A composition as claimed in claim 1, in which the protein of the MHC is from a heterologous source with respect to the cell from which the microsomes are obtained..

3. A composition as claimed in any preceding claim, in which the composition additionally comprises one or more co-stimulatory molecules.

4. A composition as claimed in claim 3, in which the co-stimulatory molecules are selected from the group consisting of B7 and IL-2

5. A composition as claimed in any preceding claim, in which the antigen is from a viral, bacterial, yeast, fungal, or protozoan origin.

6. A composition as claimed in any preceding claim, in which the antigen is an auto-antigen

7. A composition as claimed in claim 6, in which the antigen is of neoplastic cell or cell of a cancer tumour, or a normal self-protein.

8. A vaccine composition comprising a composition as defined in any one of claims 1 to 7.

9. A composition as defined in any one of claims 1 to 7 for use in medicine.

10. A method of treatment or prophylaxis of a subject, comprising the step of administering to the subject a vaccine as defined in claim 8.

11. The use a composition as defined in any one of claims 1 to 7 in the preparation of a vaccine for the prophylaxis or treatment of a disease condition.

12. A use as claimed in claim 11, in which the disease is an infection caused by a virus, bacterium, yeast, fungus or protozoan.

13. A use as claimed in claim 11, in which the disease is cancer

14. A use as claimed in claim 11, in which the disease is an autoimmune condition

15. A process for the preparation of a vaccine composition as defined in claim 8, the process comprising incubating a microsome and an antigen in the presence of a nucleoside triphosphate, and formulating the resulting preparation in an physiological diluent and optionally an adjuvant.

16. A kit of parts comprising a composition as defined in any one of claims 1 to 7 and one or more cytokines and/or adjuvants in sealed containers.

17. A kit of parts as claimed in claim 16, in which the cytokine is Il-2 or IFNγ

18. A kit of parts comprising a composition as defined in any one of claims 1 to 7 and one or more cytokines and/or adjuvants for separate, subsequent or simultaneous administration to a subject.

19. A kit of parts as claimed in claim 18, in which the cytokine is Il-2 or IFNγ

Fig. 1.

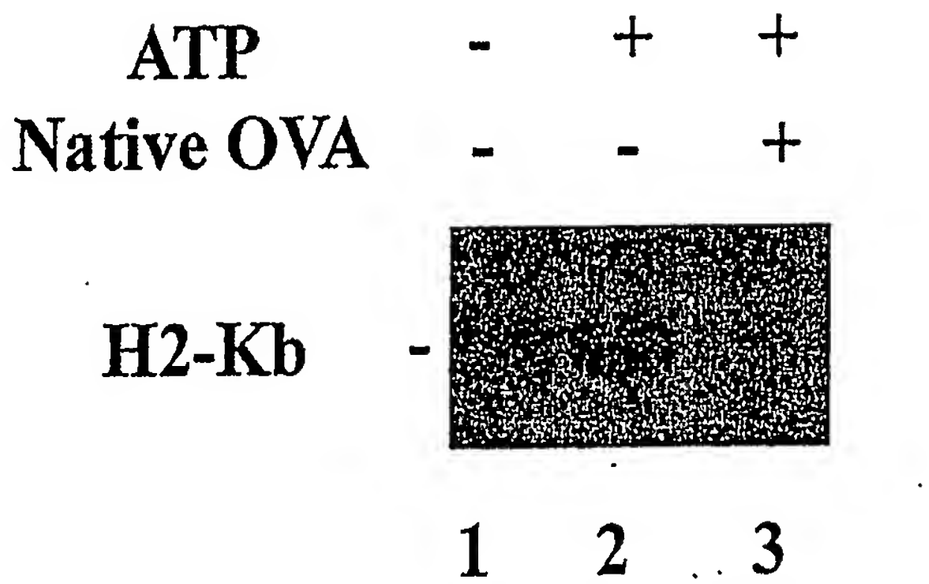
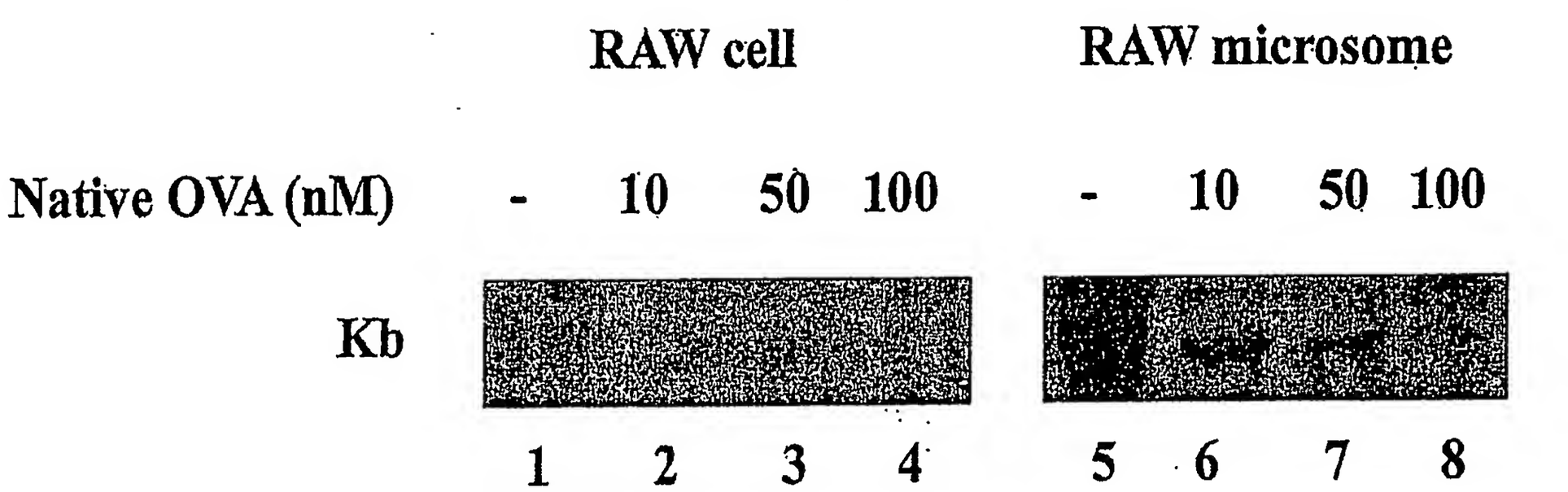


Fig. 2.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- 10 50 100
Kb
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Fig. 3.

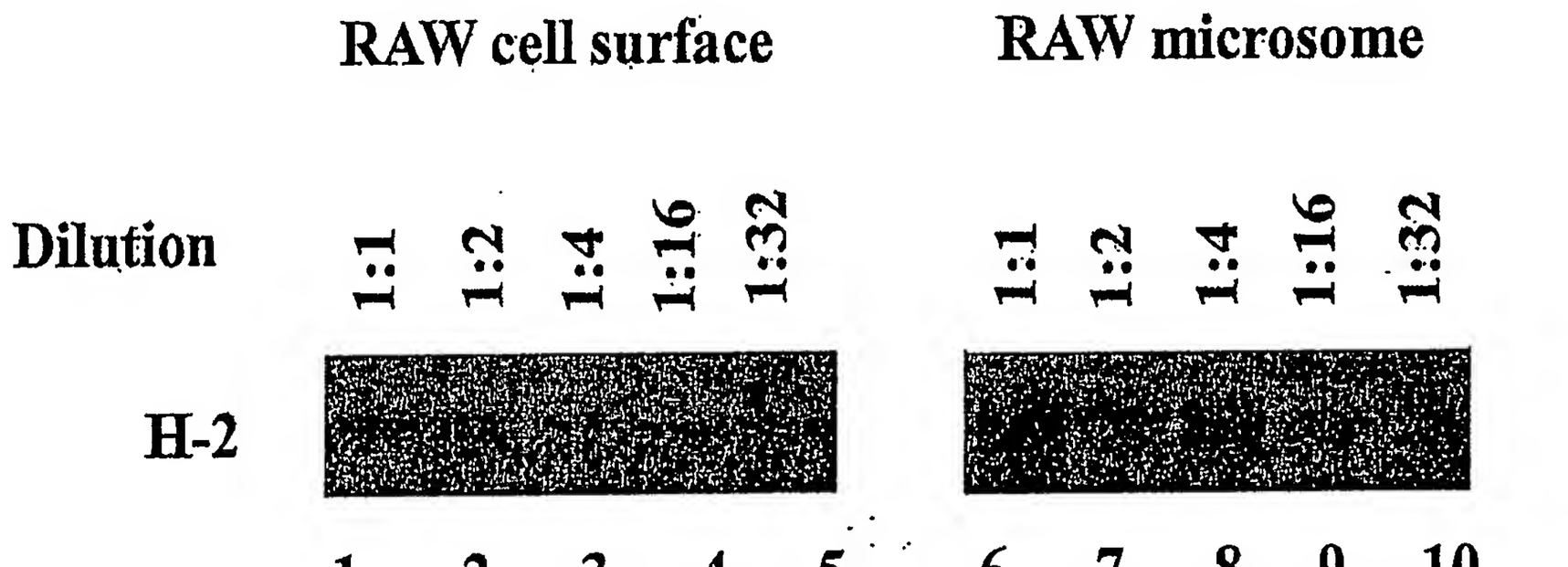
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Fig.4.

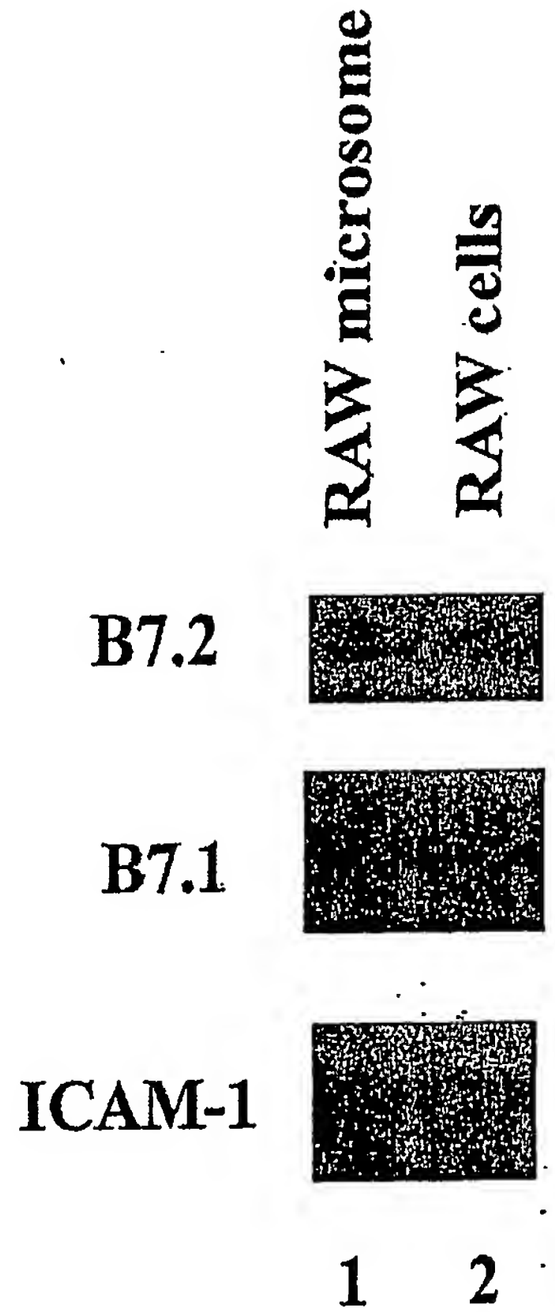
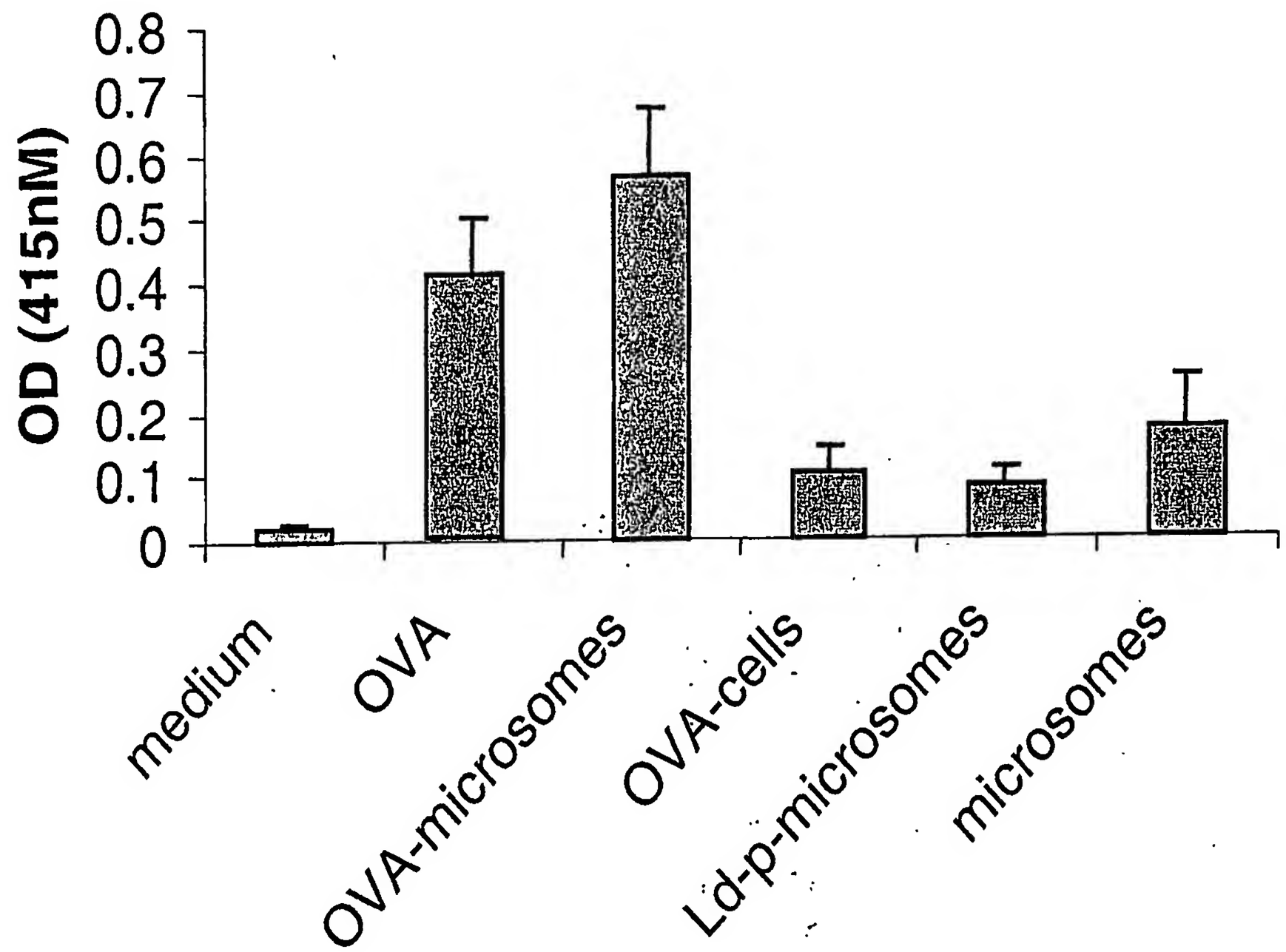


Fig. 5a.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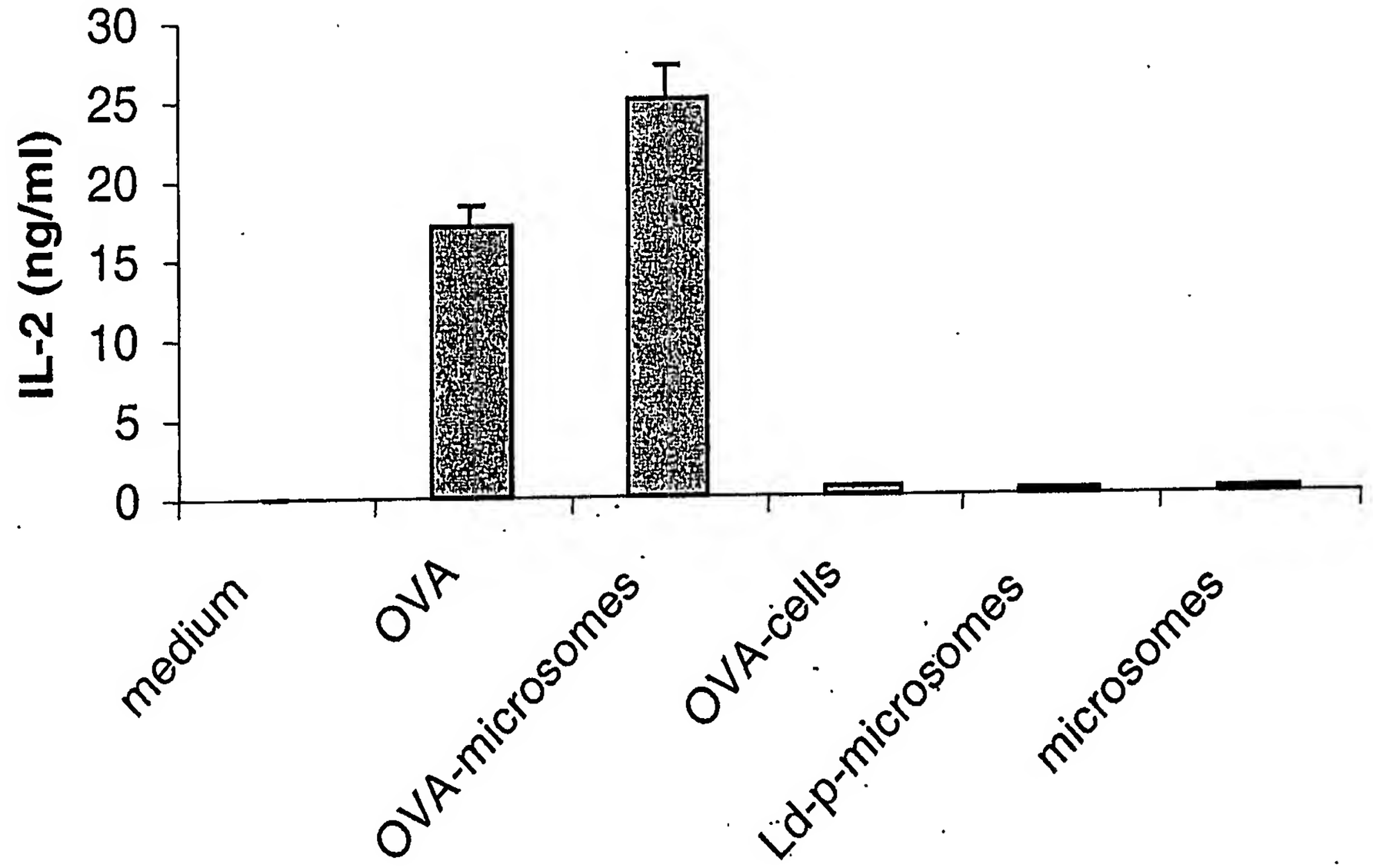
OVA-cells
Ld-p-microsomes
microsomes

Fig. 5b.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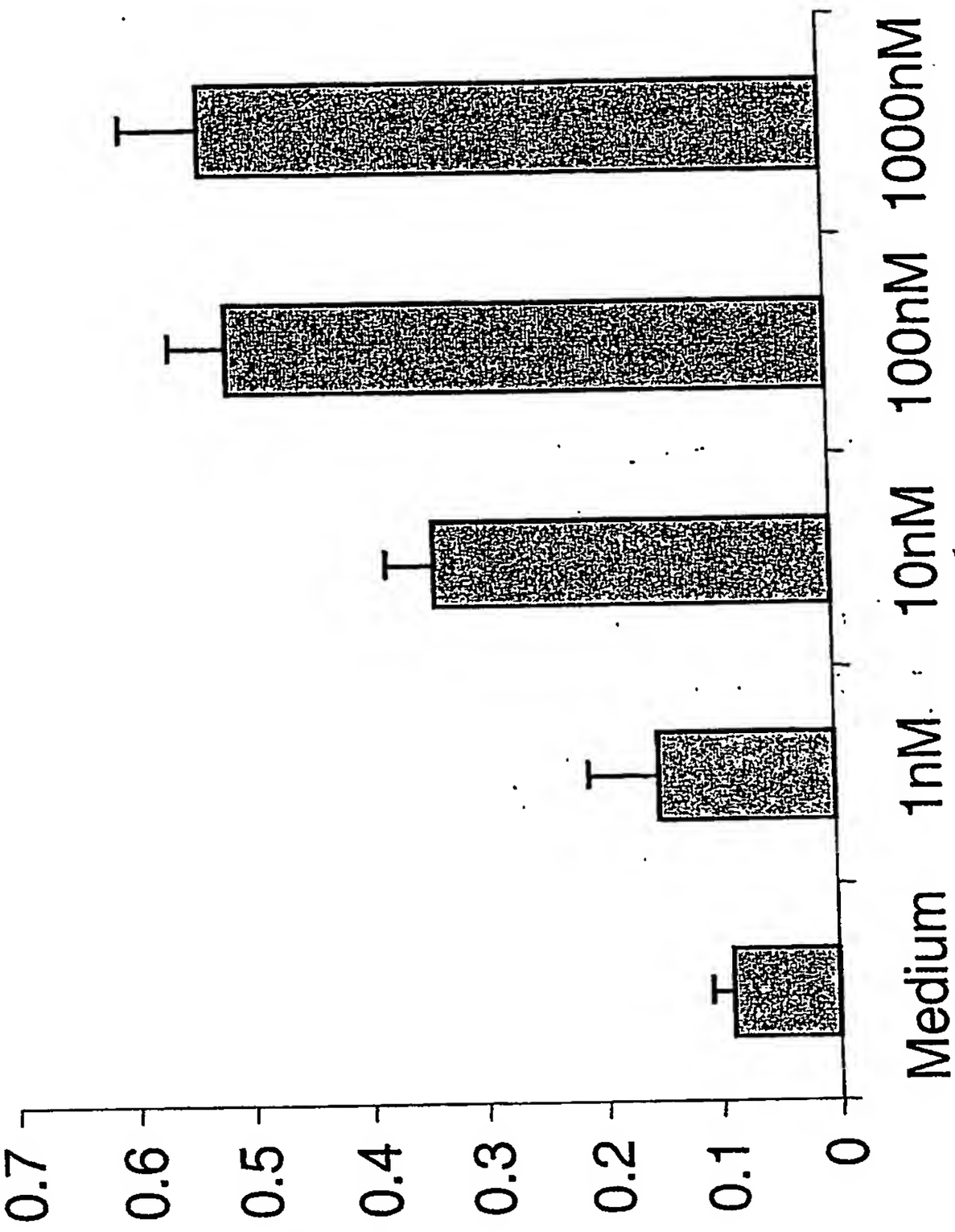

Fig. 6.

Fig. 7a.

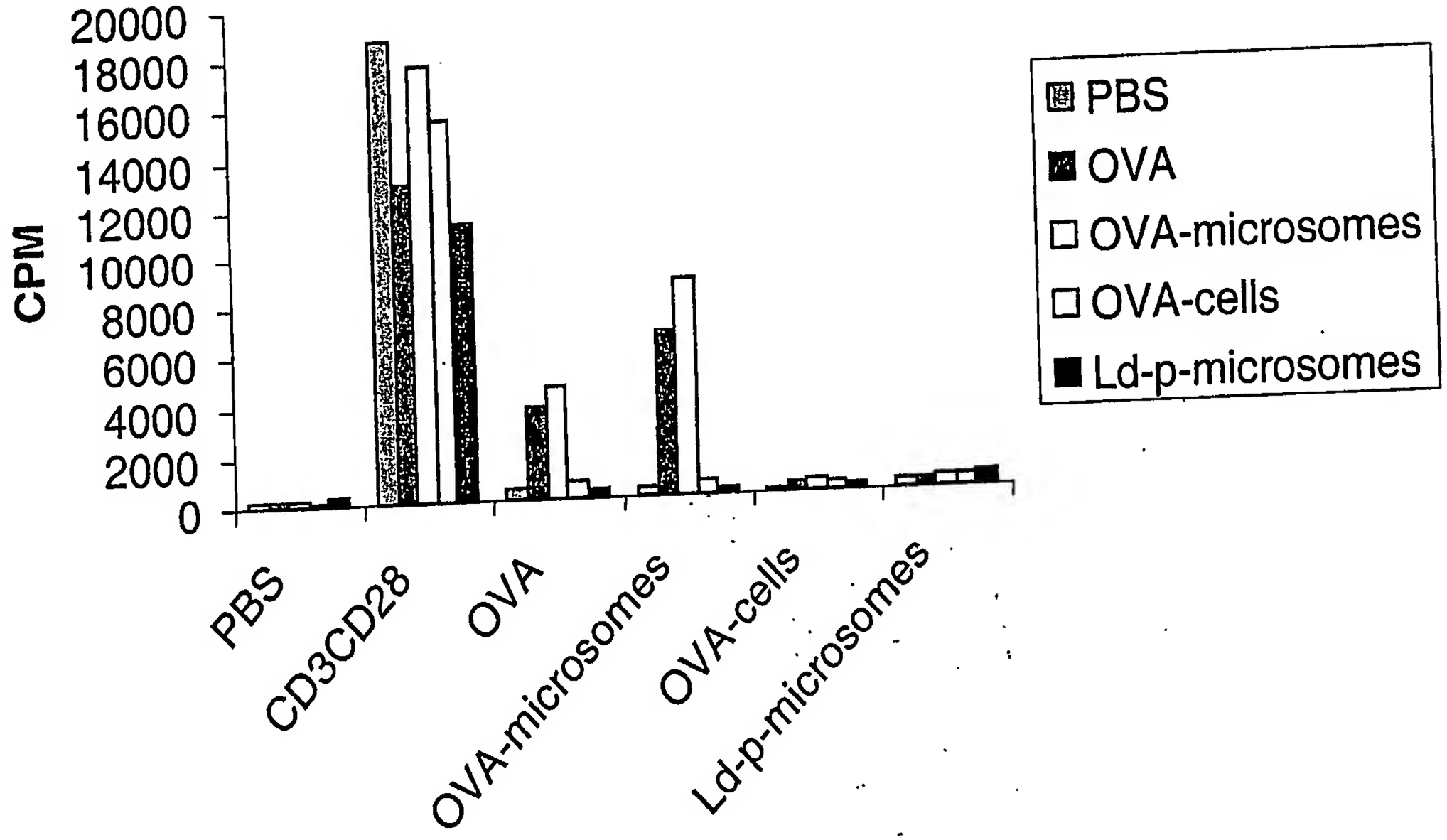
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Fig. 7b.

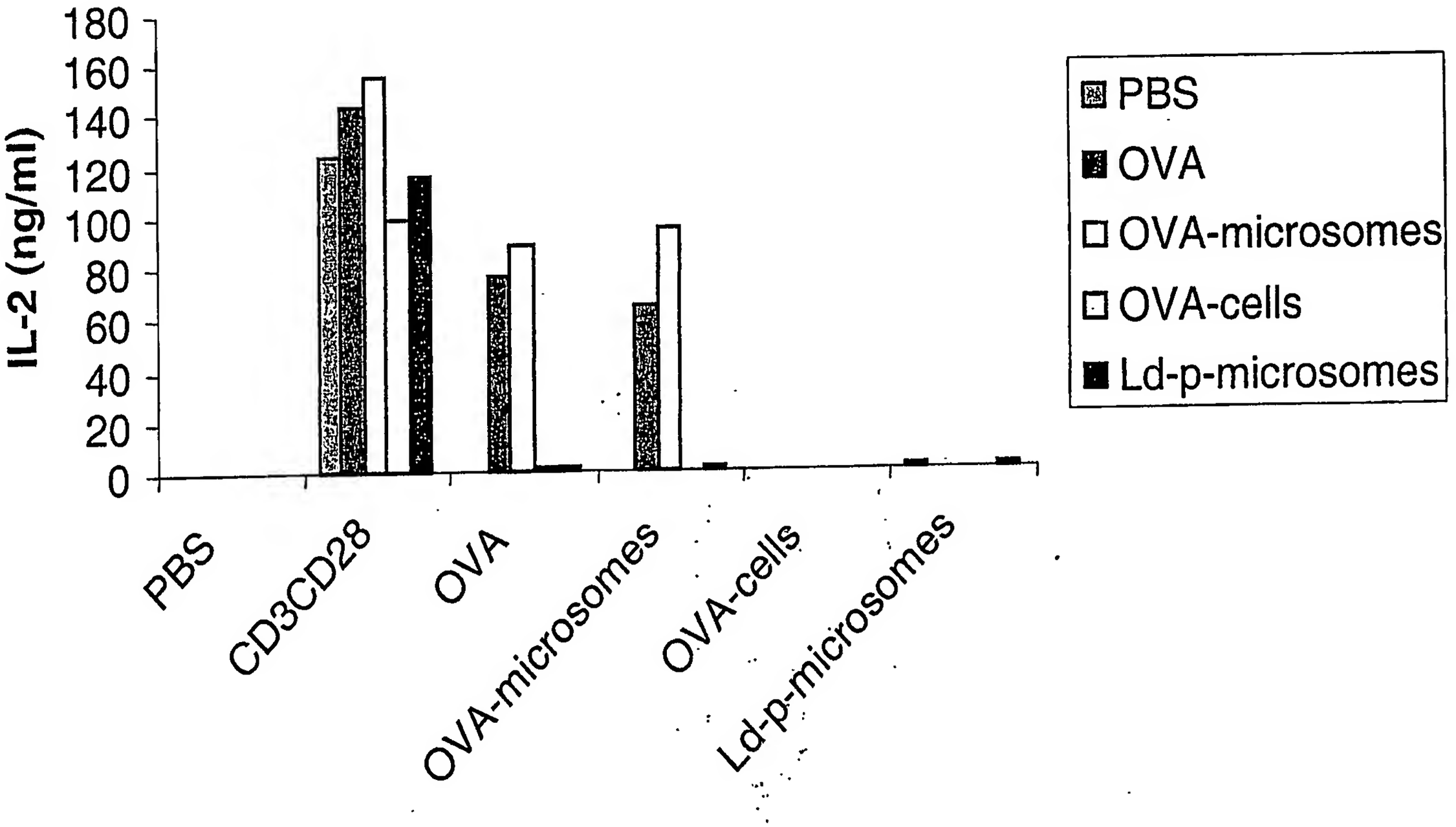
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Fig. 8.

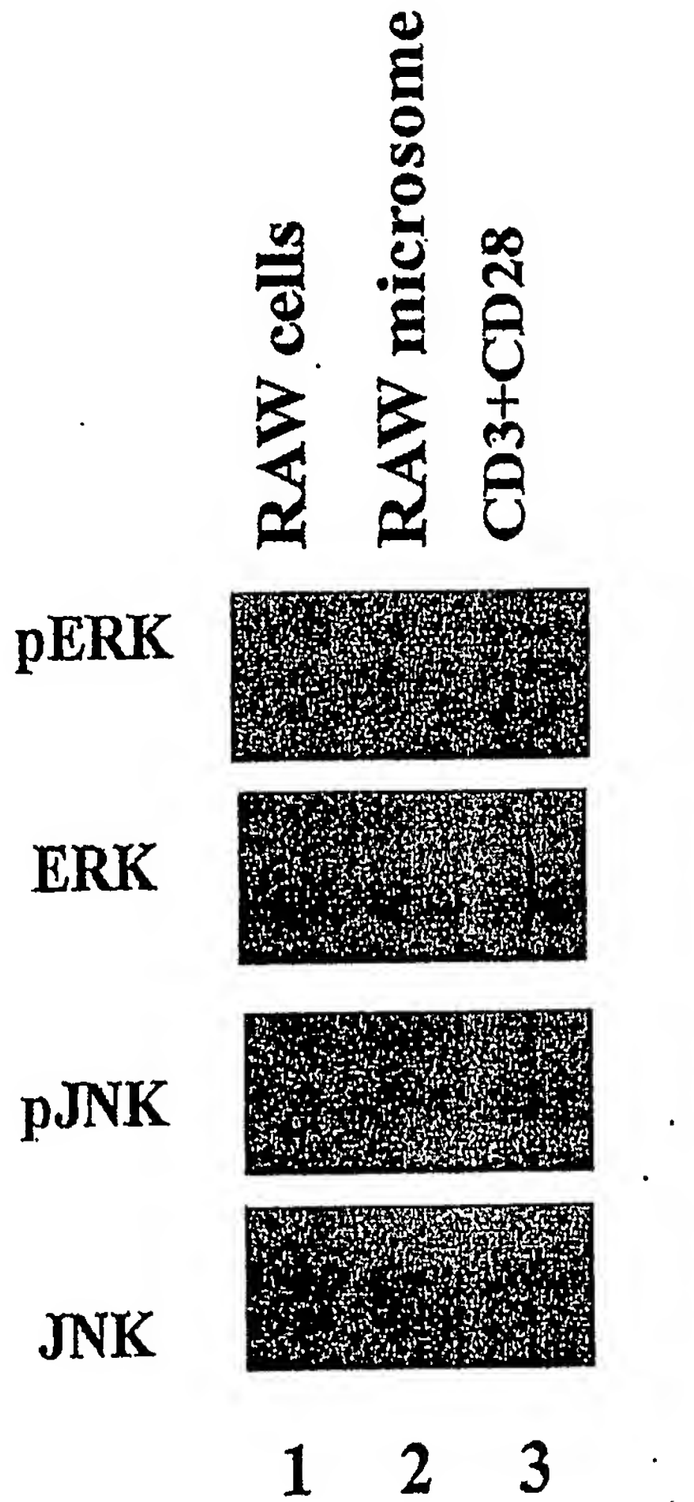

Fig. 9. Binding of 125I-SIINFEKL to Kb